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Research report

NGF gene transfer to intrinsic basal forebrain neurons increases cholinergic cell size and protects from age-related, spatial memory deficits in middle-aged rats

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Abstract

Administration of nerve growth factor (NGF) by intracerebroventricular infusion or transplantation of NGF-secreting cells to the basal forebrain improves spatial memory in aged animals. Using the adeno-associated virus (AAV) vector system, basal forebrain neurons were transduced to produce NGF ectopically for long intervals (at least 9 months). Rats received intraseptal injections of either the control vector, pTR-UF4, or the pTR-NGFmyc at 3 months of age, prior to testing their performance in the Morris water task. An age-related decrease in the acquisition of the hidden platform location was found at 12 months of age in the pTR-UF4 control group, but not in the pTR-NGFmyc group. Further, when compared to 3 month old untreated animals, the control group, but not the pTR-NGFmyc group, was impaired at 12 months of age. Concomitant to preventing age-related memory deficits, the NGF gene transfer increased cholinergic neuron size by 34% in the medial septum. This approach may therefore represent a viable therapy for age-related dementia involving dysfunction in cholinergic activity and memory, such as Alzheimer's disease.

Theme: Disorders of the nervous system

Topic: Genetic models

Keywords: Adeno-associated virus; Choline acetyltransferase; Gene transfer; Gene therapy; Morris water task; NGF

1. Introduction

Therapeutic strategies for age- and dementia-related memory dysfunction that involve trophic factors may be beneficial because they could counteract the progressive nature of aging and neurodegenerative disease. Nerve growth factor (NGF) therapy is one potential approach to protect from and potentially reverse memory deficits and neurodegeneration. Intracerebroventricular administration of NGF to 23–25 month old rats for periods of 2–4 weeks, improves spatial acquisition [7] and retention [19] in the Morris water task and improves scores of recent memory in the *T*-maze test [19]. NGF administration to the basal forebrain of aged rats via grafts of either NGF-secreting fibroblasts [5,21] or neural progenitor cells [20] is efficacious with respect to acquisition [20,21] and retention [5,20,21] of spatial learning in the Morris water task. Further, when the NGF-secreting progenitor cells were grafted to middle-aged animals, they prevented age-related deficits in memory function over a subsequent 9 month time-course [22]. In that study, the NGF mRNA derived from the graft could be detected in 5 of 9 animals probed by reverse-transcriptase PCR, 4–9 months post-grafting. Although NGF improves memory-related performance in aged animals, it is difficult to deliver to functional sites in the brain on a long-term basis.

For the purposes of human gene therapy and functional studies, an advantageous method to deliver trophic factor to the brain is the use of viral vectors (see [14, 29] for review). The major advantages of this approach relate to the localization and long-term expression of trophic factor transgenes. A single stereotaxic injection of vector can

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produce NGF protein in intrinsic basal forebrain neurons for extended intervals [12]. Acetylcholine-producing neurons in this area that project to the hippocampus are important in memory function as cholinergic lesioning impairs spatial memory [27,35] and cholinergic agonists improve memory [23,32]. The main known side effects of NGF intracerebroventricular infusion are weight loss in rats [19,37] and in humans [6] and back pain in humans [6]. These could potentially be overcome by limiting NGF expression to the areas of the populations of neurons that affect memory. Several vector systems have been used to express NGF in the brain including lentivirus [2], adenovirus [3], and adeno-associated virus [12,18]. These strategies produced significant effects on cholinergic neurons including protection from aspiritive fimbria-fornix lesioning [2,18], and increased cell size [3] and cholinergic marker expression [12].

In this study, we investigated memory-related performance in middle-aged, male Sprague–Dawley rats. Aging is associated with a progressive decline in memory function, with middle-aged rats showing impairment in measures of learning and memory intermediate compared to either young or aged rats in several studies. Aitken and Meaney [1], Kadar et al. [10], and Shukitt-Hale et al. [31] observed that spatial memory performance of middle aged rats in the Morris water task was intermediate to young and aged rats. The results from Lindner [16] also suggest a linear decline in water maze performance between 2 and 26 months of age. Modeling early versus late stage memory impairment allowed us to investigate the effects of gene transfer at a time before a broad range of age-related dysfunction has developed, including motor deficits [31] and blindness [33]. Further, the use of 7–12 month old rats allowed the opportunity to test for either therapeutic or detrimental effects of gene transfer at ages that are potentially more responsive to NGF compared to aged rats.

2. Materials and methods

2.1. Vectors

The two constructs used, pTR-UF4 and pTR-NGFmyc, retain 4% of the wild-type AAV genome, the terminal repeats, and have been described previously [12]. Expression of *gfp* is driven by the rat neuron-specific enolase promoter in both constructs. The internal ribosome entry site from poliovirus provides bicistronic expression of ngf_{myc}(derived from mouse *ngf*) and *gfp* in the pTR-NGFmyc. As previously described, full neurotrophic activity of the NGFmyc fusion protein was confirmed in PC12 cell cultures [25]. Plasmids were propagated in SURE cells (Stratagene) and CsCl-purified.

Plasmids were packaged in recombinant AAV (rAAV) by the method of Grimm et al., [8] and the rAAV vectors were purified as previously described [12,13]. Briefly, human embryonic kidney 293 cells at 70% confluence were transfected by the calcium-phosphate method with one of the AAV terminal repeat-containing plasmids in an equimolar ratio with the plasmid pDG, which provides the AAV coat protein genes and adenovirus 5 genes necessary for helper function in packaging [8]. Forty eight h after transfection, cells and media were harvested and pelleted at 3000×*g*. The pellet was resuspended in 50 mM Tris pH 8.3, 150 mM NaCl and freeze-thawed 3 times. After spinning at 3000×*g*, 1/3 volume saturated ammonium sulfate pH 7.0 was added. After precipitating for 10 min on ice, samples were centrifuged at 8000×*g*, and ammonium sulfate was added to the resulting supernatant (2/3 the original volume). After precipitating for 20 min on ice, samples were centrifuged at 17,000×*g*. The pellet was resuspended in CsCl/PBS at 1.37 g/ml density and spun for 12 h at 55,000 rpm. The middle third of the gradient was removed, diluted in the CsCl/PBS, and respun for 12 h at 55,000 rpm. The gradient was fractionated and samples positive for rAAV DNA, as detected by PCR analysis, were pooled and concentrated in Millipore Ultrafree-4 units. When the sample volume was concentrated to 100 μl, it was overlaid with 1 ml of lactated Ringer's solution and this procedure was repeated twice before collecting the rAAV stock in a final volume of 400 μl. Recombinant AAV was titered for total particles by a quantitative-competitive PCR assay. Particle titers for both of the rAAVs (copies of DNA/ml) that were used were 3×10^{12}, resulting in 6×10^{9} particles in a 2 μl injection.

2.2. Animals and surgical procedures

Male Sprague–Dawley rats (250–300 g) were weighed and anesthetized with 3 ml xylazine (20 mg/ml), 3 ml ketamine (100 mg/ml), 1 ml acepromazine (10 mg/ml) administered intraperitoneally at a dose of 0.6 ml/kg. The injection coordinates for the medial septum were 0.7 mm bregma, 0.2 mm medial-lateral, −6.8 mm dorsal-ventral [28]. Virus stocks were injected through a 27 ga. cannula connected via 26 ga. I.D. polyethylene tubing to a 10 μl Hamilton syringe mounted in a CMA/100 microinjection pump. The pumps delivered 2 μl over 15 min, and the needles remained in place at the injection site for 1 additional min. Animal care and procedures followed institutional IACUC and NIH guidelines.

Animals (15–16/vector group) were injected with rAAV at 3 months of age. Four months after, 8 animals from either vector group at 7 months of age were tested for memory-related behavior. Nine months after vector injections, the remaining 7–8 animals from either vector group at 1 year of age were tested for memory-related behavior. At the conclusion of the behavioral testing all of the animals were weighed, sacrificed, and analyzed for transgene and choline acetyltransferase (ChAT) expression. Also, untreated animals at 3 months of age ($n=6$) were tested for memory-related behavior. Animals were

assigned to groups randomly; no behavioral attributes were examined prior to testing spatial memory at 3, 7, or 12 months of age.

2.3. Morris water task

A modified procedure of Morris [26] was used as previously described [23]. The acquisition phase of the hidden platform test entailed 3 days of 12 trials/day. The circular tank was 152 cm diameter, filled 30 cm deep with opaque, milk-colored water maintained at a constant temperature of 25°C. Visual spatial cues were maintained constant during all sessions, including the trainer, who was blinded with respect to the experimental groups. Before the initial trial on the first day, the animals swam for 60 sec (or less if the platform was found sooner), and rested on the hidden platform for 30 sec. The subjects then began 12 trials per day for 3 days (run over the same 2 h period each day) followed by a 1 trial platform removal probe phase on day 4. For the 36 trials, escape latencies were measured as the time to reach the hidden platform. If the animal did not reach the platform within 1 min, animals were manually guided to the platform and remained there for 30 sec, and an escape latency of 60 sec was recorded. One day after the last trial in the hidden platform test, the platform was removed and the animals were tested in 1 trial of the probe phase lasting 1 min. A video tracking system (San Diego Instruments) was used to measure number of target crossings, time spent in the target quadrant, and swim distance and swim speed during the probe phase. The 3 month old untreated animals were tested on the same days as the 7 month old AAV-treated animals. The 12 month old animals were tested later and similar experimental conditions were adopted in all sessions.

Learning curves of the acquisition of the hidden platform location were compared by 2-way (trial by vector group) repeated-measures ANOVA. The vector groups were also compared for mean escape latency during the hidden platform test and target crossings, % in the target quadrant, and swim distance and swim speed during the platform removal test by Dunnett's *t*-tests, which correct for multiple comparisons.

2.4. Histochemistry

Anesthetized animals were perfused with 100 ml of cold phosphate-buffered saline (PBS), followed by 400 ml of cold 4% paraformaldehyde in PBS. After, the brain was removed and equilibrated in a cryoprotectant solution of 30% sucrose/PBS and stored at 4°C. Coronal sections (30 μm) of forebrain were analyzed for several markers. Native GFP fluorescence was detected using a fluorescein isothiocyanate long-pass filter. Antigen detection was performed by incubation in a blocking solution (1% bovine serum albumin/0.3% Triton X-100/PBS) with free-floating sections for 1 h at room temperature, followed by primary antibody incubation overnight at 25°C. Primary antibodies and the dilutions used in this study were: GFP (Clontech polyclonal, 1:300), myc (Santa Cruz monoclonal, 1:300), and ChAT (Chemicon polyclonal, 1:500). After, the sections were washed in PBS, and incubated with biotinylated anti-rabbit (Sigma, 1:1000) or anti-mouse (Sigma, 1:10,000) secondary antibody for 1 h at 25°C. The sections were then washed with PBS and labeled with horseradish peroxidase (HRP)-conjugated Extravidin (Sigma, 1:1000) for 1 h at 25°C. Development of tissue labeled with HRP was performed with a solution of 0.67 mg diaminobenzidine (Sigma) and 0.13 μl of 30% H_2O_2 per ml of 80 mM sodium acetate buffer containing 8 mM imidazole and 2% $NiSO_4$.

2.5. Image analysis of cholinergic neurons

ChAT-positive neuron profiles in the medial septum and the vertical limb of the diagonal band on the side of the vector injection were analyzed on samples of every 6th serial section (5 sections analyzed/animal) over a 0.9 mm rostrocaudal extent of the forebrain (within 1.2 and 0.2 bregma) in a blinded manner with respect to the experimental conditions. Sizes of ChAT-positive neurons from all of the 5 sections from 14 to 16 animals per vector group, on the side of the vector injections, were measured using the NIH Image 1.62 software in a blinded manner. Pixel^2 areas were converted to μm^2 by measuring 1 and 2 mm squares. Mean ChAT-positive cell numbers and sizes for the 2 vector groups were compared by *t*-test.

3. Results

3.1. Memory-related behavior

A significant age-related decrease in acquisition of the platform location was found in the control group. For the pTR-UF4 group, 7 month old animals acquired the platform location at earlier trial intervals compared to the 12 month old animals (Fig. 1A; $F(1, 14)=16.62$, $P<0.002$, 2-way repeated measures ANOVA). However, for the pTR-NGFmyc group, no age-related decrease in platform location acquisition was observed. Comparing the 7 month old and the 12 month old animals in the pTR-NGFmyc group did not yield a significant effect of age (Fig. 1B; $F(1, 13)=2.52$, $P=0.14$). For both vector groups, significant effects of trial interval on escape latency were found (pTR-UF4: F $(1, 35)=5.90$, $P<0.0001$; pTR-NGFmyc: $F(1, 35)=8.48$, $P<0.0001$). Significant age by trial interval interactions were not found within either vector group (pTR-UF4: F $(1, 35)=1.36$, $P=0.09$; pTR-NGFmyc: F $(1, 35)=1.24$, $P=0.17$). Also, no significant age-related differences in either vector group were found between 7 and

12 months with respect to time spent in the target quadrant and target crossings during the platform removal probe phase (not shown).

Learning curves for acquisition of the location of the hidden platform were similar 4 months post-injection of AAV in 7 month old rats injected with either pTR-UF4 or pTR-NGFmyc ($F(1, 14)=0.34$, $P=0.57$). However, a significant vector group by trial interval interaction was observed at 7 months (F (1, 35)=1.48, $P<0.05$). Performance during the probe phase was similar for the 2 vector groups at this age. When animals were tested 9 months after rAAV injections in 12 month olds, there was a trend for the pTR-NGFmyc group to acquire the location of the hidden platform at earlier trials compared to the pTR-UF4 group, although this vector group difference only approximated statistical significance ($F(1, 13)=3.54$, $P=0.08$) and no significant vector group by trial interaction was found (F (1, 35)=0.68, $P=0.92$). Similar to what was observed in the probe phase with the 7 month old animals, no vector group differences were found in the 12 month old animals with respect to time spent in the target quadrant and target crossings. Swim distances and swim speeds were similar for both vector groups in the 7 month and the 12 month old animals.

Fig. 1C shows the mean escape latencies over the 36 trials for the 3 month old untreated rats and the 2 vector groups at the 2 ages. When the mean escape latency for the 3 month old untreated rats (20.9±4.5 sec, $n=6$) was compared with the other 4 groups, a significant difference in the acquisition phase was found only in the 12 month old control, pTR-UF4 group (43.5±2.9 sec, $n=8$, $P<0.01$, Dunnett's t-test). As analyzed by 2-way repeated measures ANOVA, there was a significant age-related decrease in the mean escape latencies in the pTR-UF4 vector group (7 month old: 23.7±3.8 sec; 12 month old: 43.5±2.9 sec, $n=8$/interval, $P<0.01$, Dunnett's t-test), but not the pTR-NGFmyc vector group ($n=7$–8/interval).

3.2. Transgene expression

Nine months after receiving rAAV injections, animals were analyzed for GFP and NGFmyc expression in order to demonstrate the stability of transgene expression. All of the animals in both vector groups demonstrated significant levels of GFP expression by native fluorescence (not shown) and immunostaining (Fig. 2A, B). GFP signal was not found either by fluorescence or immunostaining in uninjected animals or in non-transduced tissue (e.g., the left edges of panels 2A, B). The injections (0.2 mm medial to the midline) produced bilateral expression of GFP. While no myc immunoreactivity above background staining was observed in the pTR-UF4 group (not shown), all of the animals in the pTR-NGFmyc group had significant levels of septal neurons expressing immunoreactivity specific for NGFmyc (Fig. 2C), as observed previously [12]. NGFmyc

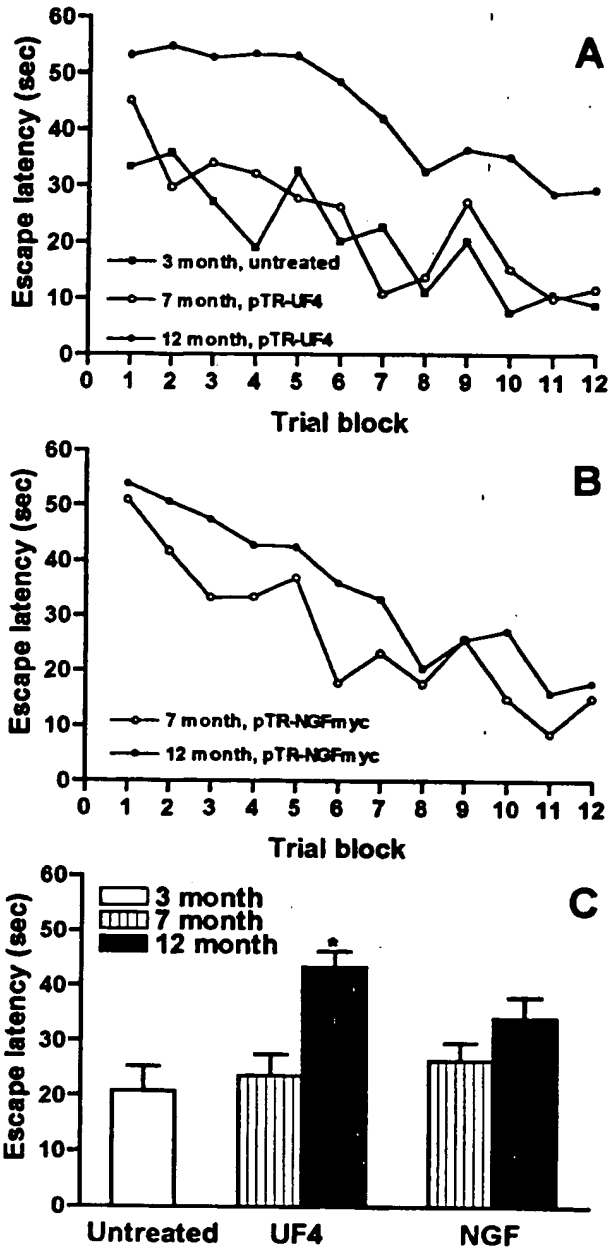

Fig. 1. Acquisition of the hidden platform location in 7 and 12 month old animals receiving either the pTR-UF4 or the pTR-NGFmyc rAAV. Animals ($n=7$–8/vector group/interval) were injected with 6×10^9 particles of either rAAV at 3 months of age. Data are shown in blocks of 3 trials. (A) For the control pTR-UF4 vector group, a significant age-related decrease in the acquisition of the hidden platform location was found between 7 and 12 months of age by 2 way repeated measures ANOVA ($F(1, 14)=16.62$, $P<0.002$). (B) For the pTR-NGFmyc group, acquisition of the hidden platform location was similar at 7 and 12 months of age. (C) By comparing the escape latencies (mean±S.E.M.) over the 36 trials, a significant age-related impairment was found in the 12 month old control pTR-UF4 group relative to either the 7 month old pTR-UF4 control group ($P<0.01$, Dunnett's t-test; $n=8$/interval) or the 3 month old untreated animals ($P<0.01$, Dunnett's t-test, $n=6$). In contrast, the 12 month old pTR-NGFmyc group was not impaired compared to either the 7 month old pTR-NGFmyc group or the untreated animals.

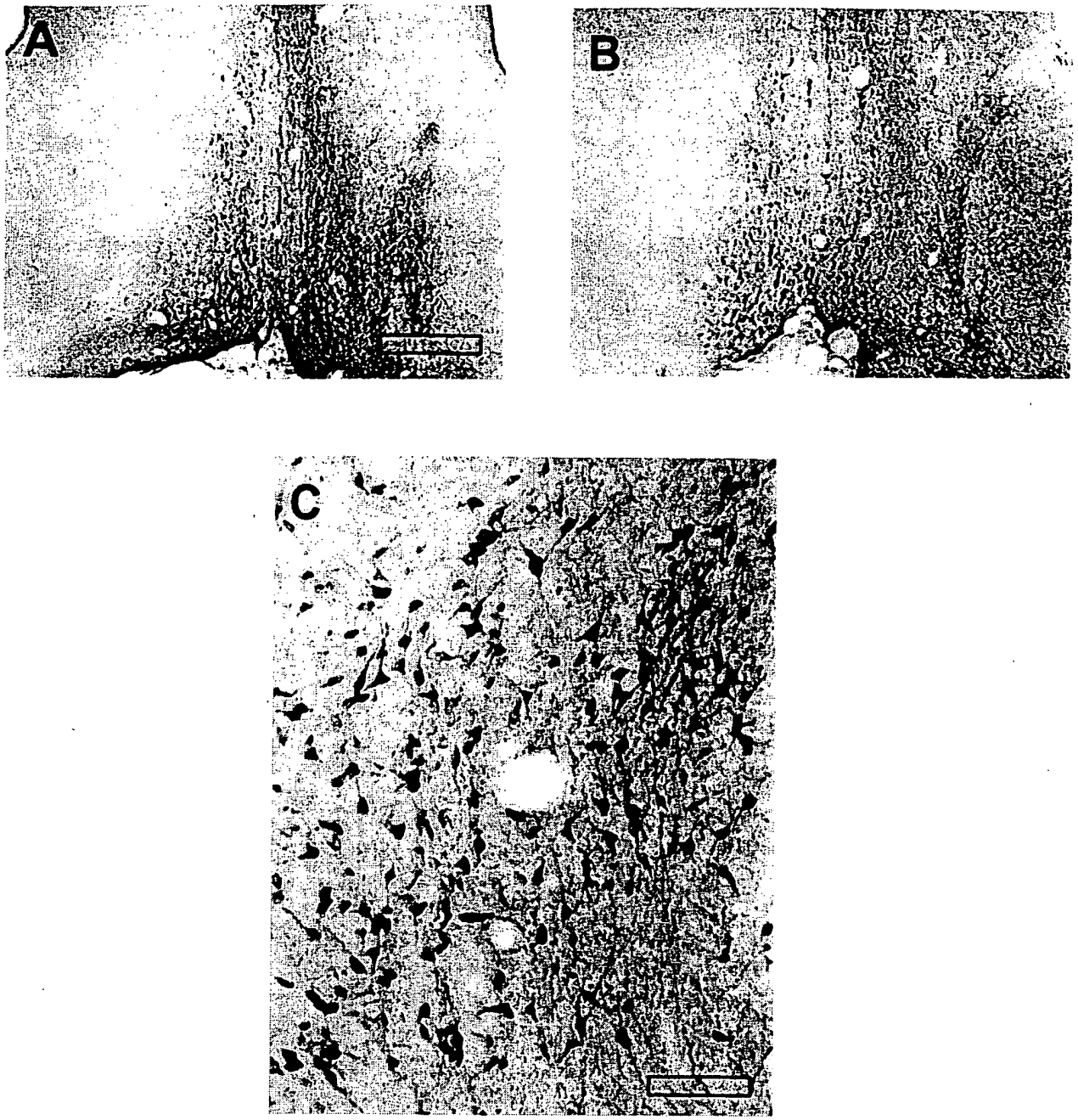

Fig. 2. Recombinant AAV-mediated transgene expression in the basal forebrain at 9 months post-injection. (A) Example of GFP immunostaining in the pTR-UF4 group, bar=500 μm. (B) Example of GFP immunostaining in the pTR-NGFmyc group. A, B, same magnification. (C) Example of NGFmyc immunoreactivity from the pTR-NGFmyc group, midline to the left of the panel, bar=80 μm. Immunoreactivity for myc above background staining was not observed in the pTR-U4 group as shown previously [12].

staining was found in the cytoplasm of neuronal cell bodies and processes although not in nuclei. We previously showed that myc-tagged trophic factor derived from the vector co-localized with the bicistronically-expressed GFP [11] and that the rAAV transduced both cholinergic and non-cholinergic neurons in the septal region [12]. The long-term expression of NGFmyc in the basal forebrain did not result in body weight differences between the 2 vector groups at the conclusion of the study (pTR-UF4=501±9 g; pTR-NGFmyc=483±10 g; n=15–16/group).

3.3. Cholinergic neuron size

No difference was observed with respect to the number of ChAT-positive neurons detected by immunostaining in the medial septum and vertical limb of the diagonal band on the side of the vector injection (pTR-UF4 group: 40.0±2.6 cells/section; pTR-NGFmyc group: 43.6±2.9 cells/section; $n=14–16$/vector group). However, the cholinergic neurons were significantly larger in the pTR-NGFmyc group compared to the pTR-UF4 group (Fig. 3). In the medial septum and vertical limb of the diagonal band, cholinergic neurons exposed to the pTR-NGFmyc rAAV had, on average, 34% greater cross-sectional area compared to cholinergic neurons in the control group (pTR-UF4: 166±10 μm^2; pTR-NGFmyc: 222±18 μm^2; $n=14–16$/vector group; $P<0.02$, t-test).

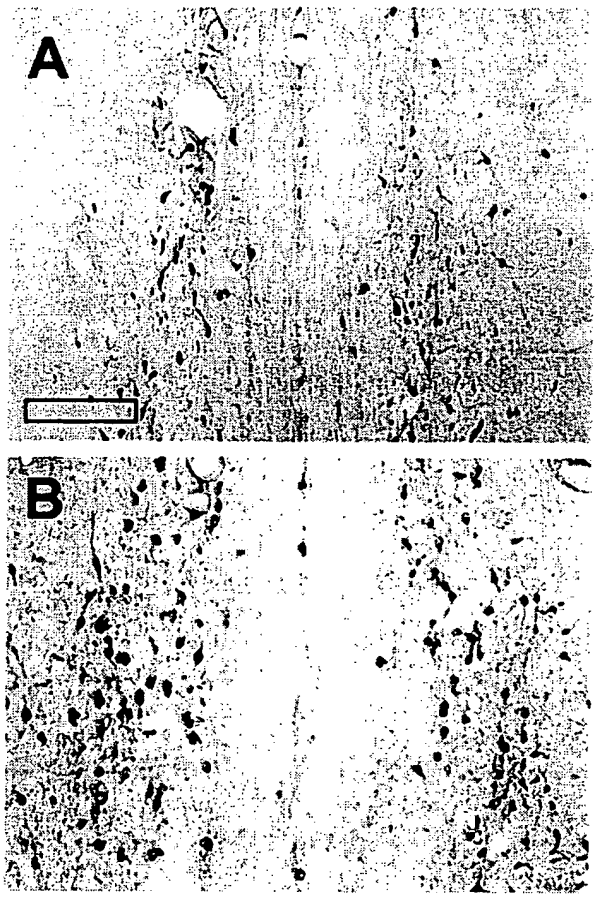

Fig. 3. Recombinant AAV-mediated NGF somatic gene transfer to the basal forebrain increases cholinergic neuron size (9 months post-injection). (A) Example of ChAT-positive neurons in the septal area from the pTR-UF4 group, bar=200 μm. (B) Example of ChAT-positive neurons from the pTR-NGFmyc group. A, B, same magnification. Measuring the cross-sectional area of septal cholinergic neurons on the side of the injection (left side of the panels) demonstrated a significant 34% increase in the pTR-NGFmyc group compared to the pTR-UF4 control group at 9 months post-injection ($P<0.02$, t-test).

4. Discussion

NGF somatic gene transfer was effective for a long interval in the basal forebrain with respect to expression levels and impact on cholinergic neuron size. The 12 month old, middle-aged rats treated with control vector in this study were significantly impaired in acquiring the location of the hidden platform compared to 7 month old control rats, as well as untreated 3 month old rats. The longer escape latencies in the 12 month old group was not due to motor impairment as they had similar swim distances and swim speeds in the platform removal probe phase as the 7 month old group. Consistent with previous reports, the 12 month old, control rats were significantly impaired relative to younger animals in acquiring the hidden platform location [1,10]. However, no change was seen in the probe phase of the Morris task (i.e., % time in the target quadrant and number of target crossings). This was perhaps due to a greater sensitivity of acquisition versus retention measurements to detect impairment in intermediate-performing animals [1,10,31].

In contrast to the control vector group, the 12 month old, middle-aged rats treated with NGF vector were not significantly impaired relative to either 3 month old untreated or 7 month old NGF-treated rats. The data suggest that the long-term NGF administration was protective from an age-related decline in spatial memory. Because littermates were randomly assigned to the groups, we believe that each group received a similar population of learners, although we cannot rule out the possibility that the 12 month old NGF vector group received a disproportionate number of the better learners at the outset of the study. Further, the lack of change in the 7 to 12 month olds in the NGF group may have reflected a slight impairment at 7 months of age that rendered similar responses at the 2 time points. Indeed, detrimental effects of NGF administration on spatial acquisition in young animals has been found [19]. We did observe a significant vector group by trial interval interaction when comparing the acquisition of 7 month old control and NGF vector groups, which suggested that the NGF 7 month old group exhibited lower rates of learning (slopes of the acquisition curves) than control. However, both the 7 and 12 month old NGF-treated groups had similar escape latencies compared to 3 month old untreated animals, whereas the platform acquisition of the 12 month old control group was impaired, which provided evidence that the NGF gene transfer protected from age-related spatial memory impairment.

The NGF expression did not appear to reduce body mass as was observed after intracerebroventricular infusion [19,37], at least relative to the pTR-UF4 control vector

after 9 months. The lack of effect on body mass found in this study compared to NGF peptide infusions could be due to several features of the NGF administration including dosage, localization, and duration. In terms of potential non-specific effects of the control vector, pTR-UF4, there was no evidence of toxic effects in the septal area (e.g., Nissl, neuron-specific, and ChAT staining) in this or a previous study [12]. In addition, memory-related behavior in the 7 month old pTR-UF4 group was similar compared to the untreated 3 month old group. Further, bilateral injections of the pTR-UF4 rAAV and GFP expression in the substantia nigra for periods of 2 months, did not alter either basal or amphetamine-stimulated locomotor activity, relative to vehicle injections (unpublished).

Transgenes continued to be expressed (GFP in the pTR-UF4 vector group and GFP and NGFmyc in the pTR-NGFmyc vector group) in every animal tested at the 9 month conclusion of the study. This is consistent with earlier reports showing long-lived expression of trophic factors via AAV injections into the basal forebrain [12] or the substantia nigra [13]. More importantly, the transgenic NGF continued to exert neurochemical effects on cholinergic neurons after 9 months. Many studies suggest that trophic effects of NGF on cholinergic neurons underlie the positive effects on behavior in aged [5,7,9,20–22] and lesioned animals [17,35], although other studies have questioned this hypothesis [4,15]. NGF administration increases cholinergic activity in neonatal [24] and 3–6 month old animals [5,9,12,36], although NGF administration to young animals does not improve memory [5,19]. It is possible that the presence of quiescent, metabolically-slowed cholinergic neurons is necessary to permit NGF's memory-enhancing effects.

In conclusion, long-term NGF gene transfer localized to the basal forebrain neurons in adult animals increased cholinergic neuron size while apparently protecting the animals from developing age-related deficits in memory function. This may be of particular relevance to Alzheimer's disease gene therapy because: (1) the basal forebrain, NGF receptor-expressing, cholinergic neurons are among the first rendered hypofunctional during Alzheimer's disease pathogenesis [34]; and (2) localized NGF expression proximal to basal forebrain neuron cell bodies could overcome the deficient retrograde transport of target-derived trophic factors associated with the disease [30]. Improved methods for delivering genes to localized areas of the brain on a long-term basis like those described here and previously [2,3,12,13,18] may make trophic factor therapies both less toxic and more efficacious.

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